Conjugate of a tissue non-specific alkaline phosphatase and dextran, process for its production and use thereof.

The invention concerns a conjugate of a tissue non-specific alkaline phosphatase and dextran, a process for the production of such a conjugate and its use.

Alkaline phosphatases (AP, EC 3.1.3.1) belong to a ubiquitous family of dimeric metalloenzymes which catalyse the hydrolysis of phosphomonoesters under alkaline conditions with release of inorganic phosphate (McComb et al., (1979), Alkaline Phosphatases, Plenum Press, New York). One can distinguish between four isoenzymes in humans: i) placenta-specific AP, ii) germ cell specific (placental) AP, iii) intestinal AP and iv) the tissue non-specific AP (tns-AP) (Harris, H., Clin. Chim. Acta 186 (1990) 133-150). The production of tns-AP is strongest in the liver (LAP), kidney (KAP) and bones (BAP) (Moss, D.W., Clin. Chem. 38 (1992) 2486-2492) and is the most frequent AP isoform in serum (Mulivor, R. A., et al., J. Lab. Clin. Med. 105 (1985) 342-348). The differences between LAP, KAP and BAP are due to different posttranslational O-glycosylation patterns (Miura, M., et al., Ann. Clin. Biochem. 31 (1994) 25-30) which also results in different specific activities (Nosjean, O., et al., Biochem. J. 321 (1997) 297-303) although their amino acid sequences are essentially identical (Weiss, M. J., et al., J. Biol. Chem. 263 (1988) 12002-12010). Furthermore Nosjean et al. have shown that the N-glycosylation of tns-AP is essential for its enzymatic activity. Consequently tissue non-specific AP is a mixture of different glycosylated APs.

The gene for human tns-AP was already cloned in 1986 (Weiss, M.J., et al., Proc. Natl. Acad. Sci. USA 84 (1986) 7182-7186). It codes for a protein consisting of 524 amino acids with a 17 amino acid long N-terminal signal sequence and a C-terminal GPI anchor sequence with which the protein is anchored in vivo to the outside of the plasma membrane (Hooper, N.M., Clin. Chim. Acta 266 (1997) 3-12). Although the DNA sequence of human tns-AP has been known for a long time, only the expression of a recombinant, biologically active enzyme in eukaryotic cells such as COS-1 (Fukushi, M., et al., Biochem. Biophys. Res. Commun. 246 (1998) 613-618) or insect cells infected with baculovirus (Oda, K., et al., J. Biochem. (Tokyo) 126 (1999) 694-699) has been previously reported.

The heterologous expression of proteins in prokaryotes such as Escherichia coli is a technology that is frequently used for the safe and cost-effective production of recombinant proteins. The expression of eukaryotic proteins in prokaroytes has two characteristics: i) prokaryotes such as E. coli do not carry out a number of post-translational modifications such as glycosylations that are typical for eukaroytes and ii) eukaryotic proteins expressed in prokaryotes are often present in the form of poorly soluble, biologically inactive protein aggregates (inclusion bodies, IBs) (Makrides, S.C., Microbiol. Rev. 60 (1996) 512-538; Balbas, P., Mol. Biotechnol. 19 (2001) 251-267). The latter can be converted back into an enzymatically active form by known methods (Lilie, H., et al., Curr. Opin. Biotechnol. 9 (1998) 497-501). For this purpose the IBs are firstly dissolved by adding a chaotropic agent such as urea and renatured by dialysis or dilution in a chaotrope-free buffer. A method for renaturing a placenta-specific alkaline phosphatase is described in the prior art (US 5,434,067).

Although the physiological role of alkaline phosphatase is largely unknown, the determination of its enzymatic activity is one of the routine analyses in clinical diagnostics. A change in the AP activity in serum is a diagnostic marker for a large number of clinical conditions such as hypophosphatasia and hyperphosphatasia (Silve, C., Curr. Opin. Rheumatol. 6 (1994) 336-339), diseases of the liver, the bile ducts and sepsis (Maldonado, O., et al., J. Clin. Gastroenterol. 27 (1998) 342-345; Wiwanitkit, V., BMC Fam. Pract. 2 (2001) 2) and also bone diseases (Romagnoli, E., et al., Clin. Chem. Lab. Med. 36 (1998) 163-168). The serum contains a heterogeneous mixture of various forms of AP. As already stated above, the ratio of LAP, KAP and BAP which together form tns-AP also varies from patient to patient since the ratio of the individual forms of AP in serum is dependent on the type and severity of the individual disease of the patient.

For this reason it is difficult to provide suitable reference and standard samples. Serum pools consisting of sera from patients with normal AP values and increased AP values are usually used; but preparations of normal LAP or BAP are also used. The reference samples used for the determination of alkaline phosphatase in human serum or plasma are also derived from animal sources such as cows or pigs, from human cell lines or human placenta. This is associated with various disadvantages: The isolation of enzymes from animal or human tissue is associated with a high risk of infection (HIV, BSE) and is technically very complicated. Due to the lack of alternatives the International Federation of Clinical Chemistry and Laboratory

Medicine (IFCC) presently recommends the use of a tns-AP isolated from porcine kidneys as a reference enzyme (Tietz, N.W., et al., J. Clin. Chem. Clin. Biochem. 21 (1983) 731-748).

The object of the present invention is to provide a preparation of an AP which can be used as a reference in clinical diagnostics and can be produced reproducibly and in a simple manner.

The invention concerns a conjugate of a tissue non-specific alkaline phosphate (tns-AP) and dextran obtainable by reacting unglycosylated tns-AP with activated dextran in aqueous solution, stopping the reaction and isolating the conjugate from the solution.

It was surprisingly found that a conjugate according to the invention is enzymatically active, has the properties of a tns-AP and is therefore particularly suitable as a standard in AP tests. Moreover, the measured AP activity of the conjugate according to the invention in the reagent which is recommended by the IFCC (Tietz et al., supra) does not significantly depend on the buffer concentration.

IFCC reagent and reaction conditions:

Temperature	30 ± 0.05°C
pH (30°C)	10.40 ± 0.05
2-amino-2-methyl-1-propanol buffer	0.35 mol $\cdot$ 1^{-1}
4-nitrophenyl phosphate	10.0 mmol $\cdot$ 1^{-1}
magnesium acetate	2.0 mmol $\cdot$ 1^{-1}
zinc sulphate	1.0 mmol $\cdot$ 1^{-1}
N-hydroxyethylethylenediaminetriacetic acid (HEDTA)	2.0 mmol $\cdot$ 1^{-1}
volume fraction of the sample	0.0196 (1:51)

Surprisingly the same activities for the standard according to the invention were measured in a buffer range of 0.35 – 0.90 mol/l in the IFCC test. With respect to other properties the standard according to the invention behaves like a control serum in the IFCC test. In constrast, comparative experiments show that a

conjugate of unglycosylated tns-AP and glucose (glucosylated tns-AP) is unsuitable as a standard.

A tissue non-specific alkaline phosphatase (tns-AP) is understood according to the invention as an alkaline phosphatase which can be isolated in a glycosylated form from human liver, bones or kidney (EC 3.1.3.1). The nucleotide sequence of tns-AP is described by Weiss et al., 1986 supra. According to Nosjean et al., supra tns-APs from liver, bone and kidney only differ with regard to their glycosylation but not with regard to their amino acid sequence.

In a preferred embodiment a tns-AP is used as an unglycosylated tns-AP to produce the conjugate according to the invention which can be obtained by recombinant expression of a nucleic acid coding for tns-AP in a prokaryotic cell, preferably in E. coli, and optionally after naturation. Such processes for producing recombinant proteins in prokaryotes are known from the prior art (cf. Lilie et al., supra).

It is also preferred to use a dextran for the conjugate having an average molecular weight of 10 – 500 kDa. However, it has turned out that the molecular weight of the dextran that is used only has a very slight influence on the properties of the inventive conjugate. Dextran can be coupled to tns-AP by known methods. For this the dextran is firstly activated, preferably by periodate oxidation of dextran, cyanylation with CNBr or activation with CDAP (1-cyano-dimethylaminopyridinium tetra-fluoroborate). Subsequently it is coupled by incubation preferably at room temperature (cf. e.g. Andersson, A., et al., Int. J. Cancer 47 (1991) 439-444; Holmberg, A. and Meurling, L., Bioconjug. Chem. 4 (1993) 570-573; Lovqvist, A., et al., Cancer Biother. 8 (1993) 345-356; Olsson, P., et al., Int. J. Cancer 56 (1994) 529-537; Sjostrom, A., et al., Int. J. Cancer 70 (1997) 383-389). After stopping the reaction, preferably with an amine reagent, the conjugate can be isolated by known purification methods such as chromatographic methods. Dextran is covalently bound to the unglycosylated tns-AP at random positions by this method which results in a heterogeneous mixture of conjugates of dextran and tns-AP. The suitability of the conjugate according to the invention is ensured by the fact that reproducible conditions are adhered to for the production with regard to temperature, activation reagent, ratio of activation reagent to dextran, ratio of unglycosylated tns-AP to activated dextran, average molecular weight of the dextran, incubation time and stop reagent. The suitable reaction

conditions according to the invention can, however, also be varied over a wide range and are uncritical.

The reaction is preferably carried out at room temperature and for about one hour. CDAP or CNBr are preferably used as activation reagents. The weight ratio of activation reagent to dextran is preferably 1:2 to 1:20.

It is preferable to use activated dextran in an excess in order to produce the conjugate according to the invention preferably in a molar ratio of 1:2 to 1:500, particularly preferably 1:10 to 1:500 (unglycosylated tns-AP to dextran).

Another subject matter of the invention is a process for producing a conjugate by reacting unglycosylated tns-AP with activated dextran by incubation in an aqueous solution, stopping the reaction and isolating the conjugate from the solution. The incubation period is preferably about one hour. The reaction is preferably terminated by adding an amine reagent such as ethanolamine.

In a preferred embodiment unglycosylated tns-AP which has been obtained by recombinant expression of a nucleic acid coding for tns-AP in a prokaryotic cell is used to produce the conjugate according to the invention.

A dextran having an average molecular weight of 10 – 100 kDa is particularly preferably used, the dextran is activated with CDAP, and unglycosylated tns-AP and activated dextran are used for the said reaction in a molar ratio of 1:10 to 1:500.

Another subject matter of the invention is the use of an unglycosylated tns-AP to produce a conjugate according to the invention consisting of unglycosylated tns-AP and dextran.

Another subject matter of the invention is the use of a conjugate according to the invention as a standard in a method for the quantitative determination of alkaline phosphatase. Such methods are described for example by Tietz et al., supra.

The following examples, publications, the sequence protocol and the figures elucidate the invention further, the protective scope of which derives from the

patent claims. The described methods are to be understood as examples which still describe the subject matter of the invention even after modifications.

<u>Description of the figures:</u>

Figure 1 restriction map of human tns-AP

Figure 2 plasmid map of pBKShuap 11

Figure 3 restriction map of pelB-APN

Figure 4 plasmid map pQ Epel BAP

Figure 5 refolding kinetics of unglycosylated tns-AP

Figure 6 elution profile of dextranized tns-AP (the figure shows the elution profile of dextranized AP (dextran of 40 kDa) in comparison to native AP)

Figure 7 activity profile of dextranized tns-AP (dextran of 40 kDa) (x: results with serum samples; Δ: results with standard according to the invention; ∇ = results with control sera (PNU = Precinorm U, Roche Diagnostics GmbH, Mannheim, PPU = Precipath U, Roche Diagnostics GmbH, Mannheim)

Figure 8 activity profile of dextranized tns-AP (dextran of 10 kDa, 40 kDa, 60 kDa, 188 kDa, 400 kDa = AP 1 10 000, etc.) in comparison with serum samples (= human sera) and control sera (PNU = Precinorm U, Roche Diagnostics GmbH, Mannheim, PPU = Precipath U, Roche Diagnostics GmbH, Mannheim = controls)

Figure 9 activity profile of dextranized tns-AP (40 kDa) compared to serum samples, to glucosylated tns-AP, control sera (PNU, PPU) and unmodified, nonglycosylated tns-AP (native AP)

Example 1

Cloning the human tns-AP gene

This section describes the isolation and cloning of the gene for human tns-AP and the construction of a fusion gene with a PelB signal sequence suitable for the expression in Escherichia coli. All DNA amplification and cloning techniques used for this are well known to a person skilled in the art and are described in Sambrook, J., et al., "Molecular Cloning: A Laboratory Manual" (1989), Eds. J. Sambrook, E.F. Fritsch and T. Maniatis, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.

Firstly the complete gene sequence for human tns-AP as well as the directly neighbouring 5′ and 3′ regions were isolated by means of the so-called polymerase chain reaction (SEQ ID NO: 3 and 4), fig. 1) from a human liver cDNA bank using the oligonucleotides apNup (SEQ ID NO: 1) and apCdw (SEQ ID NO: 2). Subsequently the PCR product was digested with the restriction endonucleases EcoRI and BamHI and ligated in an expression vector that had been cleaved with the same enzymes. The resulting plasmid is shown in figure 2 (pBKShuap11).

The fusion gene used for the expression was subsequently constructed by the following three steps: [I] A synthetic gene section was constructed by gene synthesis which fused a region encoding the PelB signal sequence to a part of the region that encodes the tns-AP gene (positions 102-503 of SEQ ID NO:3). The synthesis was carried out in a 3-step PCR reaction by means of eight (SEQ ID NO: 5-12) oligonucleotides overlapping by 20 bp, wherein in the first step gene segments of the primer pairs uppel/dwpel (fragment 1), apn1_up/apn1_dw (fragment 2), apn2_up/apn2dw (fragment 3) and apn3_up/apn3_dw (fragment 4) were prepared. In the second step fragments 1 and 2 were used as the template to synthesize fragment 5 in which the oligonucleotides uppel and apn1_dw served as primers and the fragments 3 and 4 were used to prepare fragment 6 in which the oligonucleotides apn2_up and apn3_dw served as primers. In the third step fragments 5 and 6 were used as the template to synthesize the final synthetic gene pelB-AP_N (SEQ ID NO: 13, fig. 3) in which the oligonucleotides uppel and apn3_dw were used as primers. Identical amount of primers and fragments were used for the synthesis in each of the three steps of the synthesis.

[II] A section of the htns-AP gene (bp 102-1561 of SEQ ID NO: 3) was amplified using the oligonucleotides mhuapQEup (SEQ ID NO: 14) and mhuapQEdw (SEQ ID NO: 15) as primers in a polymerase chain reaction using the plasmid according to figure 2 as template. The oligonucleotide mhuapQEdw removes the sequence coding for the last 20 amino acids of htns-AP and adds the sequence AGATCTTAGTAAGGATCCAGAT (SEQ ID NO: 18) to the 3′ end of the gene.

[III] The synthetic gene pelB-AP_N was digested with the restriction endonucleases EcoRI and BstEII, the gene segment from step [II] was digested with the restriction endonucleases BstEII and BamHI and ligated with the plasmid pQE60 (Qiagen, Hilden, Germany) that had been digested with the restriction endonucleases EcoRI and BamHI. Equal amounts of the DNA fragments used were used for the ligation reaction. The resulting fusion gene or fusion protein was named pelB-tns-AP-deltaGPI (SEQ ID NO: 16 and 17), the resulting expression plasmid in which the expression of the pelB-tns-AP fusion protein is under the control of the IPTG-inducible T5 promoter is named pQEpelBAP (fig. 4).

Example 2
Expression of the pelB-tns-AP gene in Escherichia coli

E. coli K12 was transformed with the expression plasmid from example 1 pQEpelBAP and the resulting strain was cultured in LB medium containing ampicillin. The pelB-tns-AP fusion protein was expression after addition of IPTG in the middle of the logarithmic growth phase. The cells are harvested by centrifugation after a suitable expression phase (3-12 h).

Example 3
IB isolation, solubilization and refolding

Buffer 1: 10 mM Tris-HCl, pH 8.0

Buffer 2: 8 M urea, 10 mM DTT, 100 mM Tris-HCl, pH 8.0

Buffer 3: 200 mM Tris-HCl, 40 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 9 mM GSH,
4 mM GSSG, 40 % (w/v) glycerol, pH 8.0

(DDT: dithiothreitol, GSH: reduced glutathione, GSSG: oxidized glutathione)

IB isolation

The tns-AP fusion protein is formed intracellularly in two forms: i) a small proportion (> 5 %) is present as a soluble, biologically active enzyme the activity of which can be determined by the method described by Bretaudiere & Spillmann (1984) Methods of Enzymatic Analysis, VCH, 75-82); ii) the major proportion is produced in the form of enzymatically inactive IBs. These IBs have to be isolated before solubilizing, renaturing, purifying and modifying the protein. For this the cells are taken up in buffer 1 and lysed by means of high pressure dispersion. Afterwards the IBs are isolated by several centrifugation and washing steps (in buffer 1).

Solubilization

The IBs are solubilized by stirring continuously for 2 hours at room temperature in buffer 2 containing 25 mg IBs (wet weight) per ml buffer 2. Subsequently non-solubilized protein is removed by centrifugation to prepare a clear solubilisate.

Refolding

The protein is refolded in buffer 3. For this the clear solubilisate having a final concentration of 9.6 μg/ml (protein determination according to Bradford, M.M., Analytical Biochemistry 72 (1976) 248-254) is transferred dropwise into buffer 3 while stirring constantly. This process (pulsing) is repeated 12 times at intervals of 24 hours. The enzymatic activity is determined in the refolding mixture using p-nitrophenyl phosphate as the substrate according to the method of Bretaudiere & Spillmann, ((1984) Methods of Enzymatic Analysis, VCH, 75-82) in each case 24 h after addition of the clear solubilisate; the result of a typical refolding reaction is shown in fig. 5. The refolding mixture is centrifuged 24 hours after the last addition of the clear solubilisate in order to remove insoluble protein aggregates, afterwards the active tns-AP is located in the supernatant.

Example 4

Purification and dextranization of the pelB-tns-AP fusion gene

Buffer 4:	20 mM Tris-HCl, pH 8.0
	2 mM $MgCl_2$
	0.1 mM $ZnCl_2$
	100 mM NaCl

The supernatant from example 3 containing the active tns-AP is firstly concentrated by ultrafiltration over an ultrafiltration membrane made of regenerated cellulose having an exclusion size of 10 kDa. In order to avoid loss of tns-AP by unspecific binding to the membrane, this can firstly be incubated for 24 h in a 1 % bovine serum albumin solution.

Subsequently the mixture is dialysed by flow dialysis for 24 h against buffer 4. The conductivity of the dialysis buffer is adjusted to 13.2 mS/cm, the flow rate is 20 liters buffer 4/h. The protein aggregates that form after dialysis are removed by centrifugation.

The dialysate is then concentrated to about a tenth of the initial volume by an ultrafiltration as already described above. Subsequently the protein content of the solution and the activity of tns-AP is determined as described. The protein pretreated in this manner can then be chemically modified by reaction with dextran.

For this 1 g dextran T-40 (average molecular weight 40 kDa) is dissolved in 20 ml distilled water and cooled to 4°C. After adding 200 mg CDAP bromide, 800 µl of a 0.6 M triethanolamine solution is added dropwise to the solution. The pH of this solution is adjusted to 8 with 1 M KH_2PO_4 (solution 5).

For dextranization 1.5 mg tns-AP is incubated with 300 µl solution 5 for 1 h at room temperature. The reaction is then stopped by addition of 12.5 µl of a 1 M ethanolamine solution and incubated again for 30 min at room temperature. This is followed by a 24 hour dialysis against buffer 4. The result of the dextranization is checked by gel chromatography on a TSKG 5000 PWXL column (Tosohaas) using 200 mM potassium phosphate buffer, pH 8.0 as the mobile buffer; a typical elution profile of the tns-AP before and after dextranization is shown in fig. 6. The

enzymatic activity of tns-AP after dextranization is ca. 70-90 % of the activity before the dextranization.

In an analogous manner tns-AP is coupled to dextran with an average molecular weight of 40 kDa, 60 kDa, 188 kDa and 400 kDa.

Example 5

Evaluation of dextranized tns-AP in a clinical activity test

The refolded, dextranized protein is evaluated with regard to its suitability as a reference enzyme by a method comparison in two of the buffer systems published by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Tietz, N.W., et al., J. Clin. Chem. Clin. Biochem. 21 (1983) 731-748). This test is carried out at 37°C using p-nitrophenyl phosphate as the substrate. In this method comparison the activity of AP in a human serum lies exactly on the line of bisection of the x/y diagram (abscissa: activity in 350 mM AMP, pH 10.5; ordinate 900 mM AMP, pH 10.44), i.e. the AP mixture present in serum has an identical enzymatic-specific activity in both buffer systems. A suitable reference enzyme should ideally have the same properties.

The evaluation comprised testing the activity of the dextranized tns-AP from example 4, the AP activity in human sera and the AP activity in commercial control sera (PNU, PPU, Roche Diagnostics GmbH, Mannheim, Germany) in the described method comparison; they were evaluated on a Roche/Hitachi analyser (Roche Diagnostics GmbH, Mannheim, Germany). Typical evaluation profiles are shown in figures 7 and 8: The activity profile of the dextranized tns-AP is identical to that of the human serum and lies on the line of bisection of the x/y diagram, i.e. both samples have the same activity in both buffers. Hence dextranized tns-AP fulfils the requirements for an ideal control enzyme. In this case the molecular weight of the dextran obviously has no influence on the activity profile (cf. figure 8). In contrast the AP activity in the control sera lies below the line of bisection (fig. 7).

Example 6

Glucosylation of tns-AP

Tns-AP was prepared according to example 3 and purified according to example 4. It was non-enzymatically derivatized with glucose (glucosylation) by incubating

the protein in the presence of an excess of 1- α-D-glucopyranoside. The non-enzymatic glucosylation of proteins is carried out in the presence of reducing sugars on free amino groups of a protein by the so-called Maillard-Reaction (Reddy, S., et al., Biochemistry 34 (1995) 10872-10878; Thornalley, P.J., et al., Biochem. J. 344 (1999) 109-116; Singh, R., et al., Diabetologia 44 (2001) 129-146). For this 1.6 and 9.1 µmol of recombinant alkaline phosphatase in 50 mM HEPES buffer, pH 8.0 are incubated for 24 h at 4°C in the presence of a 1000-fold excess of 1-α-D-gluco-pyranoside. The glucosylation of the alkaline phosphatase is indicated by the subsequent increase in the molecular weight of the protein that is detected by means of MALDI-TOF. The enzymatic activity is determined using the method cited in example 3 which shows that the enzyme still has 90-98 % of the initial activity after the non-enzymatic glucosylation. The enzyme preparation prepared in this manner is then used and evaluated in the clinical activity test described in example 5.

The dextranized AP according to the invention lies exactly on the line of bisection. In contrast the non-dextranized AP, the glucosylated AP and the tns-AP contained in commercial control sera lie below the line of bisection (figure 9). In contrast to dextranized tns-AP, all other APs that were tested do not fulfil the requirements for an ideal calibrator.

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